Universidade de Aveiro Departamento de Biologia
Ano 2016

Andreia Filipa Afonso Magalhães

Avaliação da atividade antimicrobiana de isolados bacterianos de pele de rã de zonas urbanas

Assessment of the antimicrobial activity of bacterial isolates from frogs' skins from urban zones

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Assessment of the antimicrobial activity of bacterial isolates from frogs' skins from urban zones

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Sérgio Miguel Reis Luís Marques, (Investigador Pós-Doc) do Departamento de Biologia da Universidade de Aveiro e co-orientação do Doutor Fernando José Mendes Gonçalves, Professor Associado c/ Agregação do Departamento de Biologia da Universidade de Aveiro e do Doutor Mário Jorge Verde Pereira, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Amphibia, *Pelophylax perezi*, microbiota da pele, atividade antimicrobiana, biomarcadores, stress oxidativo, sistema enzimático antioxidante

resumo

As populações de anfíbios têm decaído ao longo dos últimos anos devido a inúmeros fatores, tais comos, a perda de habitat, a contaminação/poluição e um dos mais importantes, as doenças. Estas perdas originam também a perda de diversidade genética das espécies, podendo comprometer a sua aptidão e também capacidade de adaptação. Tendo em conta todos estes fatores, é necessário proceder à preservação das populações de anfíbios, independentemente do local em que se encontram ser contaminado, pristino, rural ou urbano. Sabendo que os anfíbios de zonas urbanas podem ser uma fonte importante para a diversidade genética da espécie e que estão expostos, tal como as populações de zonas naturais, a agentes patogénicos, sendo que normalmente são populações negligenciadas a nível de proteção, urge a necessidade de as avaliar e proteger, nomeadamente contra agentes patogénicos. De uma forma geral, esta proteção é conferida de uma forma inata por estruturas ao nível da pele, que fazem parte do seu sistema imunitário. Estas são glândulas granulares responsáveis pela produção de compostos peptídicos capazes de inibir o crescimento de agentes patogénicos. Em acréscimo, a microbiota existente na pele estimula e complementa a atividade destas secreções. Com base nestes factos, este trabalho teve como objetivos: i) avaliar de que forma fatores como as estações do ano (Primavera e Outono) e o género, podem influenciar a microbiota cultivável da pele de *Pelophylax perezi* de zonas urbanas, ii) avaliar se os isolados bacterianos da pele apresentam atividade antimicrobiana e iii) avaliar o potencial dos isolados bacterianos com atividade antimicrobiana enquanto possíveis agentes probióticos, na presença de um agente patogénico. Os resultados obtidos mostraram diferenças entre locais ao nível das espécies isoladas, sendo poucas as espécies comuns entre locais. Além disso, foi evidenciado que num total de 120 isolados, 19 possuíam atividade antimicrobiana face a *Bacyllus aquimaris* e *Aeromonas salmonicida*. Também se verificaram diferenças na atividade antimicrobiana entre estações do ano, existindo um maior número de espécies com atividade antimicrobiana no Outono. Dos isolados com atividade antimicrobiana, os três com maior atividade, *Pseudomonas rhizosphaerae*, *Pseudomonas fluorescens* e *Bacillus mycoides* foram selecionados para a segunda fase do trabalho, em que se avaliou o seu potencial enquanto possíveis agentes probióticos. Após exposição, *in vivo*, de girinos aos probióticos, em simultâneo com *A. Salmonicida*, verificou-se que estes evitavam mortalidade dos girinos, bem como diminuíam o dano peroxidativo quando comparados com os valores do agente patogénico. Dos três probióticos *B. mycoides* mostrou ser aquele com maior capacidade de estimular as enzimas antioxidantes, sendo o agente probiótico com os valores mais baixos de dano peroxidativo.

keywords

Amphibia, *Pelophylax perezi*, skin microbiota, antimicrobial activity, biomarkers, oxidative stress, antioxidant enzymatic system

abstract

Amphibian populations have declined over the past few years due to numerous factors such as habitat loss, contamination / pollution and one of the most important, diseases. These losses also result in the loss of genetic diversity of the species, which may compromise their fitness and ability to adapt. Taking all these factors into account, it is necessary to preserve amphibian populations, regardless of being found in contaminated, pristine, rural or urban sites. Given that urban amphibian populations can be an important source for genetic diversity of the species and that they are exposed, such as populations of natural areas, to pathogens, there is a need for assess and protect them against pathogenic agents. Generally, this protection is conferred in an innate way by skin structures, which are part of your immune system. These are granular glands responsible for the production of peptidic compounds capable of inhibiting the growth of pathogens. In addition, the microbiota in the skin stimulates and complements the activity of these secretions. Based on these facts, this work had as objectives: i) to evaluate how factors such as seasonality (spring and autumn) and gender can influence the cultivable microbiota of *Pelophylax perezi* skin in urban areas; ii) assess the ability of the bacterial skin isolates to present antimicrobial activity and iii) evaluate the potential of bacterial isolates with antimicrobial activity as potential probiotic agents. The obtained results showed differences between sites at the level of the isolated species, with few common species between sites. In addition, it was evidenced that in a total of 120 isolates, 19 had antimicrobial activity against *Bacyllus aquimaris* and *Aeromonas salmonicida*. There were also differences in antimicrobial activity between seasons, with a higher number of species with antimicrobial activity in the autumn. Of the isolates with antimicrobial activity, the three with the highest activity, *Pseudomonas rhizosphaerae*, *Pseudomonas fluorescens* and *Bacillus mycoides* were selected for the second phase of the study, in which their potential action as probiotic agents was evaluated. After *in vivo* exposure of the tadpoles to the probiotics, along with *A. salmonicida*, these were found to decrease the mortality of tadpoles as well as to decrease the peroxidative damage, when compared to the values obtained from the exposure to the pathogen. From the three probiotics *B. mycoides* revealed to be the one with the greatest capacity to stimulate the antioxidant enzymes, being the probiotic agent with the lowest values of peroxidative damage.

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Chapter I
General Introduction

1. General Introduction

The Amphibians are a large class of vertebrates and are grouped into three orders: *Gymnophiona*, *Caudata* and *Anura*. The order *Gymnophiona* also called *Apoda* includes caecilians or limbless amphibians. The order *Caudata* includes newts and salamanders and the order *Anura* includes frogs and toads. (Beebe, 1996; Wells, 2010). The term *Amphibia* means "double life" which refers to life in both aquatic and terrestrial habitats. Generally, during their life cycle they suffer metamorphosis passing from water dependent larval stage to a mainly terrestrial adult stage. Amphibians are cold-blooded which means they are ectothermic organisms, depending on external factors to regulate and maintain their temperature (Demas and Nelson, 2012). This factor along with the distribution of water bodies are crucial to their geographical distribution. Few are the ones that can live in extremely low temperatures. Therefore, the higher diversity of amphibians is located in tropical areas like the American Southwest and West of Africa (IUCN 2016*;* Vitt and Caldwell, 2014). These characteristics make them one of the most sensitive groups of animals to environmental alterations. In recent times we have witnessed a worldwide decline in their populations and presently they are one of the most threatened groups. According to the IUCN Red List Assessment, the order *Caudata* has a higher threatened extinction rate (49.8%) corresponding to 275 species in a total of 552. The order *Anura* from 5532, 1749 are endangered species which corresponds to a rate extinction of 31.6%. The order *Gymnophiona*, in contrast, have just 3.4% (6 species in a total of 176 are threatened). The main factors for this occurrence are habitat loss, contamination / pollution, infectious diseases and invasive species (IUCN 2016).

Even suffering worldwide decline, with diseases being one of the main factors (Stuart et al., 2004), amphibians have structures that are part of their innate immune system and help them in their defense. One of these structures and which is common to all of them is their skin. It is one of their most important organs being used for respiration and osmoregulation and providing chemical protection against the

environment**.** This organ is rich in granular and mucous glands that are widespread on the head, body and limbs (Rollins-Smith, 2001). Their role determines their density and location. Mucous glands are the most abundant and are essentially located under the dorsum. They play an important role in maintaining the moisture of the outer surface of the skin through the production of mucous substances. Granular glands in general are more concentrated in the head and shoulders and are known to secrete antimicrobial peptides (Duellman and Trueb, 1994). These peptides have the ability to inhibit the growth of pathogenic organisms. One example is the growth inhibition of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). In *Rana pretiosa*, previously exposed to the *Bd* fungus, were collected skin secretions containing antimicrobial peptides. They verified that all peptides inhibited the growth of the chytrid fungus completely at concentrations lower than 100 μM (Conlon et al. 2016). This ability to inhibit the growth of pathogens is also observed in gram positive and negative bacteria strains (Walke et al., 2014). These results may provide new templates for the development of new antimicrobial treatments. In addition to the glands and the antimicrobial peptides they produce, the skin also shows the presence of bacterial communities that establish symbiotic relationships (McKenzie et al., 2012). The bacterial communities that are part of the microbiota, can suffer changes in its composition, being environmental conditions one of the factors that might contribute for such alterations. Furthermore, these communities also present the particularity of undergoing through changes during the development of the organisms being these host-specific (Myers et al., 2012). The microbiota also plays an important role in amphibian's immune defense system. In *Lauer et al 2008* bacteria were isolated from *Hemidactylium scutatum* skin and tested in two fungal pathogens: *Mariannaea elegans* and *Rhizomucor variabilis*. They verified that the isolates belonging to the *Bacillaceae*, *Oxalobacteraceae*, *Pseudomonadaceae*, *Flavobacteriaceae*, and *Sphingobacteriaceae* families had antifungal activity against the fungi. This type of research is important because the discovery and study of these organisms with antimicrobial ability can help on the implementation of new techniques of preservation of species. One of them is bioaugmentation which is a technique that uses

microorganism's inoculation on the skin of amphibians to increase their resistance to pathogens' exposure. There are bacteria that are usually found in amphibians and which are used as probiotics. One is *Janthinobacterium lividum* and is known for producing violacein whose compound exhibits antifungal properties inhibiting the growth of pathogens. (Becker et al., 2009). Other is *Pseudomonas reactans* and it was successfully introduced into the skin of *Plethodon cinereus* salamander. The animals which were successfully inoculated with the bacteria decreased mortality and reduction of the effects, such as loss of body mass, caused by the infectious disease Chytridiomycosis. (Harris et al., 2009). Taking into consideration this background information, and knowing that amphibians inhabiting urban areas might be an essential source of genetic variation for the respective species and also, the fact that populations in these areas are usually overlooked in terms of need for protection, our study aims at filling these gaps.

Therefore, the main objectives of this work are:

- The identification of cultivable bacterial communities present in the skin of *Pelophylax perezi* individuals from areas with different levels of urbanization and compare them between seasons (Autumn and Spring).
- The assessment of the antimicrobial activity of the bacterial isolates.
- To assess the probiotic potential of the bacterial isolates with higher antimicrobial potential.

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Chapter II

Influence of anthropogenic factors in the cultivable microbiota of amphibians and their antimicrobial potential

2. Influence of anthropogenic factors in the cultivable microbiota of amphibians and their antimicrobial potential

Abstract

Amphibians have an immune system in which their skin plays a vital role involved. This is due both to the presence of granular glands as well as the presence of bacterial communities that further protect them against potential pathogens. In recent years' amphibians' pathogens have played an important role, contributing for the mortality of large numbers of animals and even the disappearance of entire populations. The reducing number of animals contribute for a decrease in genetic variation and a consequent reduction of fitness and adaptability. In a world where natural habitat loss is also a constraining factor for amphibians, every amphibian population is essential to help maintain genetic variation and thus essential to protect. In this context amphibians from urban areas have been overlooked, despite their role as links between populations from natural sites, promoting gene flow. Taking into consideration the previously mentioned, in this study we assessed the cultivable microbiota and its antimicrobial activity from *Pelophylax perezi* from two areas with different levels of urbanization in a pre and a post-hibernation season with the double objective of screening for potential probiotic species and also to verify if seasonality could be an important factor contributing for the vulnerability to pathogens. Our results revealed nineteen isolates with antimicrobial activity and this was mainly observed in females and specially in the Autumn (pre-hibernation). Bacteria with this feature were predominantly belonged to *Pseudomonadaceae*, *Enterobacteriaceae*, *Bacillaceae*, *Comamonadaceae* and *Paenibacillaceae* families. Considering only skin microbiota, these results may indicate a potential immune vulnerability of these animals during spring (post-hibernation), and surely a great dependence of environmental conditions to modulate skin microbiota. Nonetheless the isolated bacteria with antimicrobial activity may be of potential use as probiotics for this urban areas.

Keywords: *P. perezi*, skin microbiota, antimicrobial activity

2.1 Introduction

Amphibians are found in different habitats having the ability to adapt to different ecosystems. These include both pristine and contaminated sites, as well as rural and urban sites (Marques et al., 2009; Kouamé et al., 2015) However, they have characteristics that could apparently make them vulnerable namely their extremely permeable skin, devoid of any protective structures such as feathers, scales or hair. Nonetheless, to cope with external threats, amphibians depend on it. Skin which plays an important role in respiration, osmoregulation and body temperature control (Kueneman, Parfrey, & Woodhams, 2014). Furthermore, one of its most important functions is as a physical barrier protecting from pathogens and consequent infections. This line of defense may be associated with structures present in the dermis layer, such as granular glands, that are responsible for producing antimicrobial secretions (Zug et al 2001; Stuart et al. 2008). An example of granular compounds secreted by granular glands are antimicrobial peptides which prevent infections by pathogens. Depending on the species, antimicrobial peptides (AMPs) can be produced in larger or smaller quantities and also vary in their diversity. In hybrid *P. esculentus* was verified an increased production of these peptides as well as a more efficient antimicrobial activity against *Bd* fungus compared to the parental species *P. lessonae* and *P. ridibundus*. The hybrid species by presenting a wide variety of peptides, in part acquired by the parent species, gives to the hybrid an advantage fighting against potential infections (Daum et al., 2012). In addition to the presence of the skin granular glands of amphibians are also symbiotic bacterial communities that may also have a role in immune defense of amphibians (Woodhams et al., 2014). Their stability is linked to the way they respond to an external disturbance and how they return to their normal structure after it (Shade et al., 2012). The existing microbiota in the skin will vary depending on the habitat where the amphibians are located because they dependent on both aquatic and terrestrial environments. Alterations in these environments can change the microbiota. (Kueneman et al., 2014; Lozupone et al., 2007; Costello et al., 2009). In addition to the environmental changes like temperature and factors

associated with the host, the host-microbiota ratio factor can also influence the efficiency of protection against pathogens (Grice et al., 2011). When there is an imbalance between this host-microbiota relationships, the individuals became more susceptible to diseases. In these cases, a probiotic therapy treatment through bioaugmentation can mitigate this condition (Bletz et al., 2013). In the case of amphibians, it is based on the implementation of microorganisms in the skin, extrinsic to the host, in order to increase their resistance to pathogens. An essential factor in this treatment is that a probiotic cannot interfere with the existing microbiota of the host, and must symbiotically interact with it to its protective efficacy increase. It has been showed through experiments that the addition of probiotic species can increase immunity (Woodhams et al., 2011; Bletz et al., 2013). In Becker *et al.* (2009) *Janthinobacterium lividum*, which produces an antifungal metabolite violacein, was added successfully to red-backed salamanders (red back salamander). They found that animals treated with *J.lividum* increased their concentrations of violacein in their skin, which was strongly associated with survival after experimental exposure to *B. dendrobatidis*.

Even though some works have already studied the composition of skin microbiota and its importance in disease resistance, the studies focused on amphibians from urban areas are almost nonexistent despite their potential to work as a bridge for gene flow between populations from rural or natural areas. This gene flow is essential to maintain genetic variation which, as reviewed by Allentoft and O'Brien (2010) is closely and positively correlated with fitness and adaptability of amphibians Furthermore, the influence of sampling seasonality has also been neglected. Bearing this in mind, the aim of this study was to collect and identify cultivable bacteria from *Pelophylax perezi* inhabiting urban areas and assess their variation between two sampling seasons (pre and post-hibernation), as well as their antimicrobial potential.

2.2 Materials and Methods

2.2.1 Sampling Sites

Sampling sites were selected according to the degree of urbanization. The most urbanized site is S. António Park because it is inside the city of Aveiro. The less urbanized location is the river beach Olhos de Fervença.

The river beach Olhos de Fervença (Fig. 1) is located in the parish of Cadima, in Coimbra city. The natural springs known as "Olhos de Fervença" are a source of water supply in the area but also in surrounding areas like Mira, Montemor-o-Velho and Coimbra. In 2000, this beach was established through excavation and widening of an existing bedstead of a river and the construction of a small dam. In figure 2 are the three ponds where the samples were collected (http://solagasta.com/passeio-pedonal-praia-fluvial-dos-olhos-da-fervenca-cantanhede/).

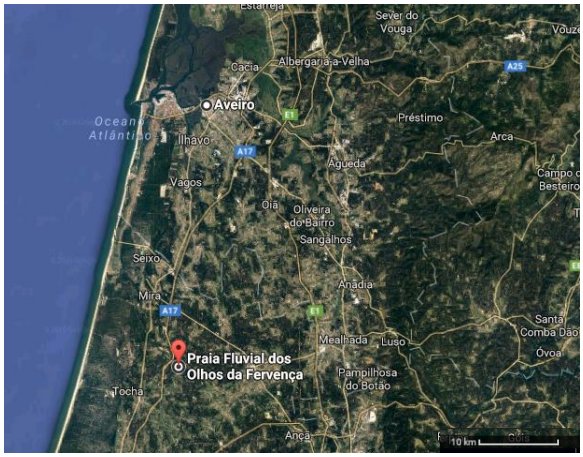

Fig. 1 river beach Olhos de Fervença location (N: 40º20'5, W:8º41'42)

Fig. 2. Ponds in Olhos de Fervença

The S. António Park (Fig. 3) is located in the city of Aveiro on the opposite site from Infante D. Pedro Park. It took advantage of Baixa da Ribeira de Santo António stream that allowed the extension of the park. (http://www.rotadabairrada.pt/irt/show/baixa-de-santo-antonio_pt_1629). In figures 4, 5 and 6 are images from the three ponds where the samples were collected.

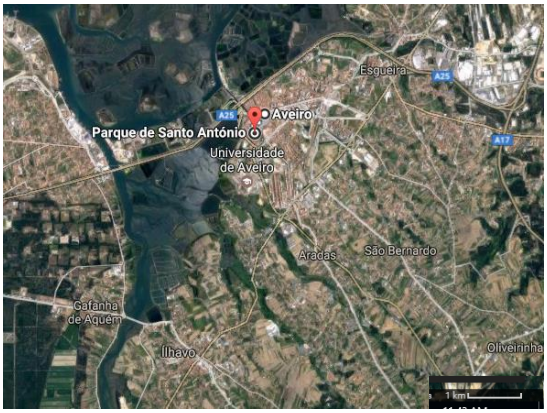

Fig. 3. S. António Park location (N: 40º38'13, W: 8º39'15)

Fig. 4. S. António Park pond 1

Fig. 5. S. António Park pond 2

Fig. 6. S. António Park location pond 3

2.2.2 Study organism

Pelophylax perezi Seoane (Fig.7) is commonly known as the Green Frog and it's distributed through the Iberian Peninsula. It is the most abundant amphibian in Portuguese territory being present in all bioclimatic regions and associated with various water bodies such as ponds and streams.

Fig. 7. *P.perezi*

Its geographical distribution is limited by the high altitude and it can be found even in humanized areas. In this species, males are smaller than females and have external vocal sacs located below the tympanum on both sides of the mouth. During the

breeding period males have black ridges on the inside of the first anterior limbs fingers. (Loureiro, Almeida, Carretero, & Paulo, 2010).

2.3 Field collection procedures and microbiota sampling

2.3.1 Frog sampling

Adult green frogs were collected in two different seasons: autumn (pre-hibernation) and spring (post-hibernation). The pre-hibernation sampling was made in November 2014 at both sites and the post-hibernation at May and June 2015. A total of 31 individuals were collected: 11 animals in pre-hibernation season (6 females in river beach Olhos de Fervença and 3 males and 2 females in S. António Park) and 20 animals in post-hibernation season (3 females and 7 males in river beach Olhos de Fervença and 5 males and 5 females in S. António Park). Animals were collected with a hand net and handled with the help of a smaller net previously washed with 70% alcohol and rinsed with sterile distilled water. Nitrile gloves were used and changed between individuals to avoid cross contamination

2.3.2 Microbiota sampling

Skin microbiota sampling was immediately made at the field after the capture of the adults. They were washed three times with sterile distilled water to remove transient bacteria from the skin (Culp et al., 2007), being collected some of the water from the last wash to confirm the effectiveness of the washes. After washing, sterile swabs were used for microbiota sampling. Each individual was swabbed three times, one per each culture medium used (TSA, TSB 1% and PCA). Each swab consisted in 3 strokes from snout to vent direction, in lateral, dorsal and ventral surfaces of the torso. The swab was slightly turned when changing within body surfaces to avoid saturating the swab with only one surface of the body. As a control for the accidental inoculation of the swab with bacteria from the air on a swab without passing through the skin was left exposed to air for thirty seconds and then swabbed in a plate with TSA medium. After finished

microbiota sampling, the length of the animal was determined using a ruler. The sex of the individuals was also determined. All swabs were immediately inoculated in the respective media. The inoculation for the solid media consisted streaking the swabs in the plate, while for the liquid medium TSB 1% the swab was placed into a 1.5 ml Eppendorf tube with 500 microliters of TSB 1% (495 microliters of distilled water: 5 microliters of TSB) and immediately preserved in a refrigerated container (4ºC) for further laboratory processing.

Growth was conducted in an incubating chamber at a temperature of 22 º C. The TSB medium, due to its liquid state at room temperature, was treated differently. Immediately after arriving at the laboratory and in a flow chamber, Eppendorf tubes were agitated in the vortex for several seconds. After this, 50 microliters of the content were pipetted and inoculated, with the help of sterile glass beads, in a Petri dish containing TSA medium. Afterwards the samples were incubated at 22 ° C in a controlled temperature room. The use of the TSB 1% intended to avoid overgrowing of fast growing bacteria, with the aim of attaining slower growing bacteria.

2.3.3 Water abiotic parameters

For every pond where animals were collected the pH (WTW330/SET-2 pH meter), dissolved oxygen (WTW315i/SET Oxi meter) and conductivity (LF 330/SET conductivity meter) were measured.

2.4 Identification of bacteria from frog skin, isolation and preservation

After bacterial growth for two-three days, each plate (TSA inoculated from TSB 1%, TSA and PCA) was verified for subsequent isolation. Colonies were distinguished according to color, size, border and texture. After observation, different colonies where isolated onto new Petri plates containing TSA. After complete isolation the bacteria isolates were preserved at -20ºC in a 20% glycerol solution for subsequent work. These steps were carried out in a laminar flow chamber.

2.5 DNA extraction

For the bacterial DNA extraction the following procedure was carried out. The isolates grew overnight in sterilized Eppendorf tubes with TSB medium (1 ml). Afterwards 100 µl were removed from the Eppendorf, placed into a new one and centrifuged at 15000 G for 15 minutes. The supernatant was then discarded and the pellet was resuspended with 40 µl of sterile water. Posteriorly it was centrifuged at 15000 G for 10 minutes and the supernatant discarded again. The pellet was then resuspended with 40 µl of sterile water and incubated at 100º C for 10 minutes and afterwards cooled on ice. New centrifugation was followed at 15000 G for 1 minute. In the end, 5 µl were run on agarose gel (1%) stained with 2 µl of SYBR® Safe DNA gel stain to check if total DNA was present. This was prepared by boiling 0.85 g of agarose in 85 ml of 1xTAE buffer (dilution of a 50x TAE stock solution).

2.6 DNA amplification and sequencing

In order to allow the identification of the bacterial isolates the amplification of the 16S rRNA genes for sequencing was performed. The PCR reactions were performed in 25 µL reactions containing 0.2 µM each primer (27F and 1492R) 1x PCR buffer, 0.2 mM each dNTP, 2 mM $MgCl_2$,1U Taq polymerase and 2 µl of cell lysate as template DNA. PCR reaction was set as follows: an initial desnaturating step, at 95 ºC, for 3 minutes, followed by 95ºC for 1 minute, 1 minute in the annealing phase, at 54 ºC, and 1 minute in extension phase at 72 ºC. This was repeated for 34x. Next was 1 minute at 72 ºC and then finished at 4 ºC. After the end of the incubation program, PCR products were analyzed by electrophoresis on agarose gel (1%) stained with SYBR® Safe DNA gel stain. The electrophoresis conditions were the follow: 45 minutes run time, 75 V and 400 Ma. Sequencing was performed by Stab Vida (Portugal) and the sequences were compared with databases, using BLASTn from the National Center for Biotechnology Information (NCBI)

2.7 Inhibition growth assays

Before carrying out the tests, it was necessary to prepare the culture medium, sterilize tweezers and small discs of paper (Whatman filter G 1) and proceed with bacteria growth (both frogs' bacterial isolates, as well as the bacteria for the test bacterial lawn). The standardized culture medium Mueller-Hinton was used in these tests. The Muller-Hinton test plates were prepared according to the disk diffusion method (Lalitha, 2008). Briefly, the bacterial cultures were left growing overnight with agitation in 15 ml Falcon tubes containing TSB medium. Then, to standardize the inoculum a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard, was used. For an absorbance value of 0.008 to 0.1 determined spectrophotometrically at 625 nm the bacterial suspension was considered to contain approximately 1 to $2x10^8$ CFU/ml. After adjusting the cell concentration, the inoculation of the plates was made. After inoculating the plate with the bacterial lawn (*Bacillus aquimaris* or *Aeromonas salmonicida*) and allowing the plate to dry, disks where placed with a minimum distance of 20 mm between them and then on each disk a different bacterial isolate was inoculated by pipetting 5 µl of the standardized inoculum. On one of the disks only TSB was pippeted as a negative control. The plate was sealed with parafilm and placed to growth for two days at 22 ° C. The plates were then examined to see whether or not an inhibition zone was visible around each disc. The choice of test agents was based, on one hand, by the pathogenic potential (*A. salmonicida*) and on the other hand by their sensitivity (*B. aquimaris*) (S. Marques, unpublished data).

2.8 Results

In the pre-hibernation season a total of 137 isolates were collected from the river beach Olhos de Fervença (Fig. 8) and 127 isolated from S. António Park. In the post-hibernation season, in S. António Park were collected 204 samples and 257 samples from river beach Olhos de Fervença.

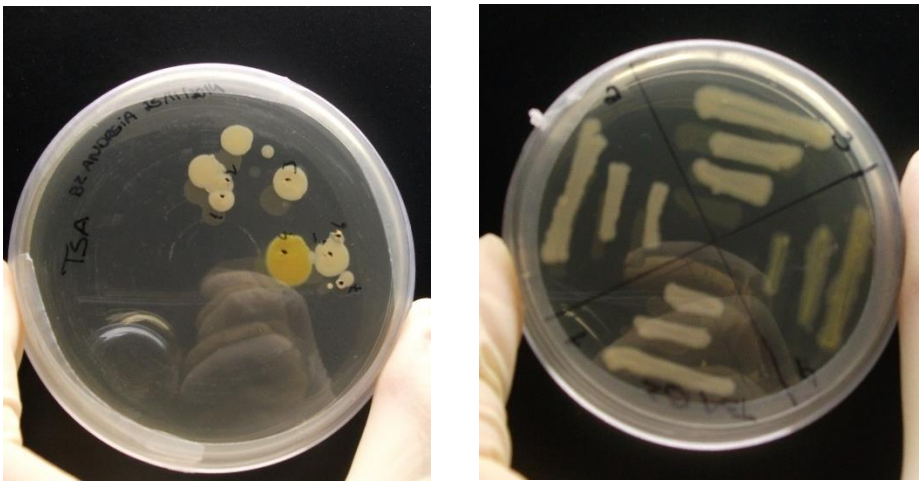

Fig. 8 Left image- Bacteria from *P.perezi* skin after growth; Right image- isolation step.

For this work and due to the number of samples in total, all isolates used in this work were among TSA medium because they were the most representative. From the autumn in river beach Olhos de Fervença were selected 38 samples and in Santo António Park 23 samples. From the spring and in river beach Olhos de Fervença were selected 26 samples and in the Santo António Park 33 samples (Table 1). The average lengths of the individuals from river beach Olhos de Fervença in the winter were from 4.8 to 6.5 centimeters. In S. António Park were between 4.2 to 4.8 centimeters. In the Spring in river beach Olhos de Fervença the sizes varied between 5 to 6.3 centimeters. In S. António Park were from 4.3 to 8.2 centimeters.

Table 1. Number of isolates per season, local, sex and with antimicrobial activity

Local		Number of isolates in females		Number of isolates in males	
		Autumn	Spring	Autumn	Spring
river beach Olhos de Fervença	Total	38	11	-	15
	Antimicrobial activity	11	-	-	1
S. António Park	Total	13	17	10	16
	Antimicrobial activity	4	-	2	1

The isolates were from *Pseudomonadaceae*, *Bacillaceae*, *Oxalobacteraceae*, *Moraxellaceae*, *Paenibacillaceae*, *Rhizobiaceae*, *Comamonadaceae*, *Enterobacteriaceae*, *Planococcaceae*, *Shewanellaceae*, *Aeromonadaceae*, *Microbacteriaceae*, *Sphingobacteriales*, *Micrococcaceae* and *Deinococcaceae*. (Table 2.)

Table 2. Identification of bacterial morphotypes obtained from *P. perezi* skin; RBO-River beach Olhos de Fervença; S- Spring; A-Autumn; M-Male; F-female; IGA-inhibitions growth assays; (+) shows antimicrobial activity in the inhibitions growth assays; (%)- percentage of identity.

Sample	**Site**	**Season**	**Sex**	**Bacteria**	**IGA**	**Family**	**(%)**
1	RBO	S	M	*Pseudomonas fluorescens*		Pseudomonadaceae	96
4	STP	S	M	*Serratia liquefaciens*		Enterobacteriaceae	97
5	STP	S	M	*Bacillus pumilus*		Bacillaceae	94
7	STP	S	F	*Massilia timonae*		Oxalobacteraceae	96
8	RBO	S	M	*Aeromonas hydrophila*		Aeromonadaceae	98
9	STP	S	M	*Bacillus megaterium*		Bacillaceae	98
12	RBO	A	F	*Pseudomonas fluorescens*	+	Pseudomonadaceae	94

13	STP	A	F	*Providencia rettgeri*		Enterobacteriaceae	94
15	STP	A	F	*Pseudomonas fluorescens*		Pseudomonadaceae	95
16	RBO	A	F	*Paenibacillus pabuli*		Paenibacillaceae	94
17	STP	A	F	*Comamonas testosteroni*	+	Comamonadaceae	93
18	STP	A	M	*Pseudomonas fluorescens*		Pseudomonadaceae	93
19	RBO	A	F	*Bacillus thuringiensis*	+	Bacillaceae	96
20	RBO	A	F	*Pseudomonas putida*		Pseudomonadaceae	92
23	RBO	A	F	*Citrobacter freundii*	+	Enterobacteriaceae	96
24	STP	A	F	*Obesumbacterium proteus*		Enterobacteriaceae	94
25	STP	A	M	*Rouxiella chamberiensis*		Enterobacteriaceae	93
26	STP	A	F	*Pseudomonas syringae*		Pseudomonadaceae	87
27	STP	A	M	*Pseudomonas putida*		Pseudomonadaceae	97
28	RBO	A	F	*Paenibacillus pabuli*		Paenibacillaceae	95
29	STP	A	F	*Rouxiella chamberiensis*		Enterobacteriaceae	96
31	RBO	A	F	*Rouxiella chamberiensis*		Enterobacteriaceae	89
32	RBO	A	F	*Pseudomonas rhizosphaerae*	+	Pseudomonadaceae	96
33	STP	S	F	*Sporosarcina sp.*		Planococcaceae;	96
34	RBO	S	F	*Aeromonas hydrophila*		Aeromonadaceae	95
35	RBO	S	F	*Flavobacterium sp.*		Flavobacteriaceae	90
36	STP	S	F	*Aeromonas hydrophila*		Aeromonadaceae	97
37	RBO	S	F	*Pseudomonas sp.*		Pseudomonadaceae	94
38	STP	S	F	*Bacillus megaterium*		Bacillaceae	97
39	RBO	S	F	*Klebsiella oxytoca*		Enterobacteriaceae	85
40	STP	S	M	*Citrobacter freundii*	+	Enterobacteriaceae	87

42	STP	S	F	*Shewanella oneidensis*		Shewanellaceae	94
43	RBO	A	F	*Skermanella stibiiresistens*		Shewanellaceae	98
44	STP	A	M	*Citrobacter freundii*		Enterobacteriaceae	95
45	RBO	A	F	*Agrobacterium fabrum*		Rhizobiaceae	95
46	STP	S	M	*Pseudomonas syringae*		Pseudomonadaceae	96
48	RBO	A	F	*Pectobacterium carotovorum*	+	Pseudomonadaceae	95
49	RBO	A	F	*Pseudomonas fluorescens*		Pseudomonadaceae	95
50	RBO	A	F	*Bacillus simplex*		Bacillaceae	95
54	RBO	A	F	*Pseudomonas alkylphenolia*		Pseudomonadaceae	96
55	RBO	A	F	*Pectobacterium carotovorum*		Enterobacteriaceae	96
58	RBO	A	F	*Pseudomonas fluorescens*	+	Pseudomonadaceae	96
59	STP	A	M	*Flavobacterium sp.*		Flavobacteriaceae	96
60	RBO	A	F	*Pseudomonas fluorescens*		Pseudomonadaceae	99
61	STP	A	F	*Pseudomonas putida*		Pseudomonadaceae	99
62	STP	A	F	*Bacillus pumilus*	+	Bacillaceae	97
63	RBO	A	F	*Pseudomonas resinovorans*		Pseudomonadaceae	97
64	STP	A	F	*Citrobacter freundii*	+	Enterobacteriaceae	95
65	STP	A	F	*Klebsiella oxytoca*		Enterobacteriaceae	98
66	RBO	A	F	*Pseudomonas fluorescens*		Pseudomonadaceae	94
67	STP	A	F	*Pseudomonas fluorescens*		Pseudomonadaceae	95
68	STP	A	F	*Acinetobacter lwoffii*		Moraxellaceae	96
69	RBO	S	F	*Buttiauxella agrestis*		Enterobacteriaceae	96

70	STP	S	F	*Flavobacterium sp.*		Flavobacteriaceae	95
71	STP	S	F	*Yersinia pestis*		Enterobacteriaceae	90
72	STP	S	F	*Aeromonas veronii*		Aeromonadaceae	96
73	STP	A	M	*Buttiauxella agrestis*	+	Enterobacteriaceae	90
75	STP	S	M	*Aeromonas hydrophila*		Aeromonadaceae	96
76	STP	S	F	*Aeromonas veronii*		Aeromonadaceae	96
77	STP	S	M	*Aeromonas hydrophila*		Aeromonadaceae	97
78	RBO	A	F	*Microbacterium sp.*		Microbacteriaceae	86
79	STP	S	M	*Acinetobacter johnsonii*		Moraxellaceae	96
80	STP	A	M	*Kluyvera intermedia*		Enterobacteriaceae	95
82	STP	S	F	*Aeromonas hydrophila*		Aeromonadaceae	95
83	STP	S	F	*Pedobacter oryzae*		Sphingobacteriaceae	94
85	STP	S	F	*Arthrobacter sp.*		Micrococcaceae	97
86	STP	A	F	*Citrobacter freundii*		Enterobacteriaceae	97
88	STP	S	M	*Arthrobacter sp.*		Micrococcaceae	95
89	RBO	A	F	*Bacillus mycoides*		Bacillaceae	96
90	STP	A	M	*Pseudomonas putida*	+	Pseudomonadaceae	96
92	RBO	A	F	*Pseudomonas syringae*		Pseudomonadaceae	95
93	STP	S	M	*Deinococcus soli*		Deinococcaceae	90
94	RBO	A	F	*Pseudomonas fluorescens*		Pseudomonadaceae	99
98	RBO	S	M	*Microbacterium foliorum*		Microbacteriaceae	99
99	STP	A	M	*Flavobacterium sp.*		Flavobacteriaceae	96
100	RBO	A	F	*Pseudomonas fluorescens*	+	Pseudomonadaceae	94
102	STP	S	M	*Flavobacterium sp.*		Flavobacteriaceae	98
103	RBO	S	M	*Flavobacterium sp.*		Flavobacteriaceae	94
104	RBO	S	M	*Delftia acidovorans*		Comamonadaceae	95
105	STP	S	M	*Bacillus boroniphilus*		Bacillaceae	96

106	RBO	A	F	*Bacillus mycoides*		Bacillaceae	91
110	RBO	A	F	*Sporosarcina sp.*	+	Planococcaceae	94
112	RBO	A	F	*Paenibacillus pabuli*		Paenibacillaceae	96
113	RBO	A	F	*Klebsiella oxytoca*	+	Enterobacteriaceae	93
115	STP	S	M	*Klebsiella oxytoca*		Enterobacteriaceae	81
116	STP	S	F	*Paenisporosarcina sp.*		Planococcaceae	95
117	STP	A	F	*Citrobacter freundii*		Enterobacteriaceae	96
118	RBO	A	F	*Bacillus mycoides*		Bacillaceae	96
119	RBO	A	F	*Microbacterium hydrocarbonoxydans*		Microbacteriaceae	96
120	RBO	S	M	*Pseudomonas protegens*		Pseudomonadaceae	98
121	RBO	S	M	*Microbacterium hydrocarbonoxydans*		Microbacteriaceae	97
124	RBO	S	M	*Bacillus mycoides*		Bacillaceae	98
125	STP	S	M	*Citrobacter freundii*		Enterobacteriaceae	97
127	STP	S	M	*Providencia rettgeri*		Enterobacteriaceae	97
129	STP	S	M	*Bacillus mycoides*		Bacillaceae	98
132	RBO	S	M	*Flavobacterium sp.*		Flavobacteriaceae	99
134	RBO	S	M	*Bacillus mycoides*		Bacillaceae	84
135	RBO	A	F	*Providencia rettgeri*		Enterobacteriaceae	84
136	RBO	S	M	*Bacillus mycoides*		Bacillaceae	98
137	RBO	S	M	*Bacillus mycoides*		Bacillaceae	99
138	RBO	S	F	*Bacillus amyloliquefaciens*		Bacillaceae	98
139	RBO	A	F	*Pseudomonas fluorescens*		Pseudomonadaceae	97
140	STP	A	F	*Pseudomonas fluorescens*		Pseudomonadaceae	97
141	STP	A	F	*Acinetobacter sp.*		Moraxellaceae	96
142	STP	A	F	*Acinetobacter johnsonii*	+	Moraxellaceae	98
146	RBO	S	M	*Bacillus mycoides*		Bacillaceae	99
149	RBO	S	F	*Aeromonas veronii*		Aeromonadaceae	88

150	RBO	S	F	*Bacillus mycoides*		Bacillaceae	99
151	RBO	S	F	*Acidovorax delafieldii*		Comamonadaceae	97
152	RBO	S	F	*Acinetobacter johnsonii*		Moraxellaceae	93
153	RBO	S	M	*Erwinia persicina*		Enterobacteriaceae	95
155	RBO	A	F	*Citrobacter freundii*		Enterobacteriaceae	97
157	RBO	A	F	*Citrobacter freundii*		Enterobacteriaceae	97
158	RBO	A	F	*Bacillus pumilus strain*		Bacillaceae	97
160	RBO	A	F	*Bacillus pumilus strain*	+	Bacillaceae	95
161	RBO	A	F	*Bacillus pumilus strain*	+	Bacillaceae	96
162	RBO	S	M	*Erwinia persicina*	+	Bacillaceae	95
166	STP	A	M	*Citrobacter freundii*		Enterobacteriaceae	95
171	RBO	S	F	*Bacillus mycoides*		Bacillaceae	98
175	RBO	A	F	*Pseudomonas fluorescens*		Pseudomonadaceae	97

A total of 19 from 120 isolates showed antimicrobial activity (Fig. 9) within which almost of them were collected at the time of pre hibernation season. There was only two samples that showed antimicrobial activity that were collected in the post hibernation season. From 19 samples, 12 belong to river beach Olhos de Fervença corresponding to females and one male (11 in the autumn and 1 in the spring respectively). Regarding the Santo António Park were 7 the samples (6 from the autumn: 4 from females and two from males, and one from one male in the spring) that showed antimicrobial activity (Table 3). They belong to *Pseudomonadaceae*, *Comamonadaceae*, *Enterobacteriaceae, Aeromonadaceae*, *Paenibacillaceae* and *Bacillaceae* families.

Fig. 9 Inhibition growth assay with visible inhibition zone; Numbers 1 to 5 corresponds to bacteria isolates from *P. perezi* skin; C is the control (TSB medium only); Base layer *B. aquimaris*.

Table 3. Bacteria species isolated from *P. perezi* skin, presenting antimicrobial activity (+) growth inhibition was observed; (−) no growth inhibition was observed.

Frog Isolate	**Test organism**	
	A.salmonicida	*Bacillus aquimaris*
Comamonas testosteroni	-	+
Bacillus thuringiensis	-	+
Citrobacter freundii	-	+
Pectobacterium carotovorum	-	+
Pseudomonas fluorescens	+	+
Bacillus pumilus	-	+
Buttiauxella agrestis	-	+
Pseudomonas putida	-	+
Paenibacillus pabuli	-	+
Acinetobacter sp.	-	+
Bacillus pumilus	-	+
Flavobacterium sp.	-	+
Pseudomonas rhizosphaerae	-	+
Bacillus mycoides	-	+

In the table below (Table 4) are the results of water analyses *in situ* (pre and post-hibernation seasons). At river beach Olhos de Fervença in the Autumn the ponds showed lower pH as well as lower conductivity related to the summer. This was also observed in S. António Park in two of the three ponds. Oxygen (%) values were lower in the Spring (post-hibernation season) compared to the winter in S. António Park.

Table 4. Abiotic parameters and the respective values in the ponds from the sampling sites between seasons.

Local	Season	pH	Oxygen (%)	Conductivity (µs/cm)	Water temperature
river beach Olhos de Fervença	**Autumn**	7.235	29	400	16.43°C
		7.284	33.6	400	
		7.267	35.97	400	
	Spring	7.48	Not measured	429	Not measured
		7.52		430	
		7.47		435	
S. António Park	**Autumn**	7.77	92.7	300	16°C
		7.25	38.6	400	
	Spring	7.87	82.3	442	24.06°C
		10.09	70.3	292	
		8.97	63.2	500	

2.9 Discussion

In this study was observed that the cultivable microbiota from the green-frog´s skin differs depending on the local, sex and season. The most frequent families were *Pseudomonadaceae*, *Enterobacteriaceae* and *Bacillaceae*. This was also observed in a study carried out by *Assis et al 2016* with *Phyllomedusa distincta* sampled in two different sites in Brazil. Comparing genders, it was observed that females presented a greater number of bacterial families than males. Families like *Oxalobacteraceae, Paenibacillaceae, Planococcaceae, Shewanellaceae, Rhizobiaceae, Sphingobacteriacea* were only found in females. In males only the *Deinococcaceae* family was found. Thus it is not expected that the difference we observed result in gender differential sensitivity to pathogens. Regarding diversity of isolates obtained for both sites, there wasn't a great difference. This was similar when comparing the diversity of isolates between seasons.

Our results also revealed that some of the isolates presented antimicrobial activity. Taking into account both bacteria used as test lawns (*A. salmonicida* and

B.aquimaris) and the information on table 3, it was observed that the antimicrobial activity was predominantly against *B. aquimaris.* Only *Pseudomonas fluorescens* obtained antimicrobial activity against both. *P. fluerescens* was isolated from a female from river beach Olhos de Fervença. In other studies, this bacterium showed antifungal (*Srivastava, S.R., 2008*) and antimicrobial activity evidenced by the production of secondary metabolites against some pathogenic bacteria strains (*Trippe et al. 2013*).

When regarding seosonality, according to our results, the antimicrobial activity was observed mainly in isolates obtained from the pre hibernation season and mostly from females. Only one male from S. António Park demonstrated this activity. Another important fact is that a slightly higher number of isolates with antimicrobial activity were retrieved from river beach Olhos de Fervença. The bacteria that showed this activity belong to *Pseudomonadaceae*, *Enterobacteriaceae*, *Bacillaceae*, *Comamonadaceae* and *Paenibacillaceae* families. Based on this it is possible to infer that the temperature and the organic matter could be a determining factor in antimicrobial activity of bacterial species. It is important to highlight that in the Autumn there were great quantities of organic matter in the soil and in water in both sites. In fact, the work carried out by Noaman et al., (2004) showed that variations on the carbon sources are capable of changing the production of antimicrobial compounds. This may indicate that seasonality might play an active role in the resistance to pathogens.

Overall our results allowed to perceive differences between the diversity of the cultivable microbiota from both sites and also within seasons. However, the most striking is that we have identified some bacteria with the ability to display antimicrobial activity, which is important if future local bioaugmentation strategies are intended to by implement. Another highlight is that seasonality might be an issue when considering amphibians' resistance to pathogens. Indeed, some common bacterial strains collected in both seasons, displayed different antimicrobial activity between seasons, with most of the antimicrobial activity observed in Autumn. It is fundamental to keep these results in mind if future bioaugmentation strategies are needed, since the season for field implementation of the strategy might play a key role on its success.

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Chapter III

In vivo assessment in *Pelophylax perezi* tadpoles of the antimicrobial potential of bacterial isolates against the pathogenic agent *Aeromonas salmonicida*

3. *In vivo* assessment in *Pelophylax perezi* tadpoles of the antimicrobial potential of bacterial isolates against the pathogenic agent *Aeromonas salmonicida*

Abstract

Amphibian pathogens are among the major causes for the recent amphibian worldwide decline. In order to deal with this problem a global endeavor has been undertaken to find ways of controlling pathogenic agents which comprise fungus, bacteria and viruses. The bioaugmentation of amphibian symbiotic bacteria with pathogen specific antimicrobial activity is one of the most promising approaches. However, the majority of the studies have been focused in the fungus *Batrachochytrium dendrobatidis*. Considering that, within bacteria, the genus *Aeromonas* is one of the main responsible for most deaths in amphibians, our work aimed at testing, *in vivo*, the effect of the exposure of *Pelophylax perezi* tadpoles to both bacterial isolates with antimicrobial activity (*Pseudomonas rhizosphaerae*, *Pseudomonas fluorescens*, *Bacillus mycoides*) and the pathogen *Aeromonas salmonicida*. Mortality, growth and the activity of enzymes of the antioxidant defense system (total glutathione peroxidase, Se-dependent glutathione peroxidase, glutathione S-transferase and glutathione reductase), as well as lipid peroxidation were used as parameters to assess the condition of the animals after exposure to the bacteria. The results showed a 33% mortality among the tadpoles exposed solely to *A. salmonicida* while mortality in other conditions was negligible, being lower than 4%. Growth, enzymatic parameters and lipid peroxidation did not reveal any statistical significant difference. Nonetheless there was a global tendency for tadpoles exposed solely to *P. rhizosphaerae*, *P. fluorescens*, *and B. mycoides* to present lower enzymatic activity when compared to organisms exposed to *A. salmonicida*. Such was also verified when comparing the isolated exposure to the probiotics with the respective joint exposure with *A. salmonicida*. Our results suggest that the sole or joint exposure to *A. salmonicida* triggers the antioxidant

defense system and that the *P. rhizosphaerae*, *P. fluorescens*, *and B. mycoides* might have an important role in avoiding cellular damage caused by the pathogenic agent.

Key words: tadpoles *P.perezi*, innate immune system, oxidative stress, antioxidant enzyme system, biomarkers

3.1 Introduction

Over the recent years amphibians' populations of have been declining due to the interaction of numerous external factors (climate change, pollution, habitat change and invasive species (Stuart et al., 2004). According to a review made by Allentoft and O'Brien (2010) the genetic variation has been found, in various studies with amphibians, to be closely and positively correlated with fitness and adaptability. Such implies that a decrease in genetic variation leads to a loss of fitness. This fact leads to an increased need to preserve amphibian populations in whatever habitat they are found, ranging from pristine habitats to contaminated ones, and from rural to urban zones. Presently, one of the main responsible factors for amphibians' declines are diseases caused by fungi, bacteria and ranavirus. Nonetheless, there are amphibian species that despite the presence of pathogens such as *Batrachochytrium dendrobatidis (Bd)* fungus are able to survive (Chen and Robert, 2011; Hayes et al. 2010). One of such examples is the work carried out by Carver and coworkers (2010) where they studied the effect of the disease Chytridiomycosis in *Litoria raniformis*. They observed that shortly after inoculation with the fungus, individuals showed signs of infection being, however, able to fully recover after a few months. This type of study demonstrates that amphibians can even survive in the presence of the fungus responsible for Chytridiomycosis and quickly recover the infection, relying solely on their immune system. The immune system comprehends the set of all the organs and cells that interact coordinately with each other and are involved in protecting the organism against potential infections. (Abbas and Lichtman, 2011) The immune responses can be distinguished in innate and adaptive. Innate immunity is present since birth and it is the first line of defense against potential pathogen. Innate immunity

is non-specific and it generates a rapid response (within minutes or hours) to prevent the entry of foreign organisms through physical barriers such as skin and mucous membranes (Abbas et al. 2008). On the other hand, adaptive immunity develops when the organism is exposed to an antigen and may take days until the response is triggered. It´s characterized by its specificity and memory effect providing an increase of effectiveness of the response to repeated pathogen exposures. In amphibians, besides its innumerous functions such as thermoregulation and respiration, the skin assumes a major role in protecting the organism against exterior aggressions, namely pathogens. It contains chemical barriers that protect from potential infections. The granular glands produce antimicrobial peptides (AMP's) which are a good example of one of the innate immune responses. AMP's can be very diverse, even within philogenically close species (Daum et al., 2012). The innate immune responses of the skin can be frequently reinforced by the action of skin bacteria (Lauer et al., 2007, 2008). One of such cases is the antifungal metabolite, violacein, produced by the bacterium *Janthinobacterium lividum* (Becker et al., 2009). It has been documented that the inoculation of this bacteria in salamanders increases their survival to infection by Bd. In fact, *J. lividum* has been used in recent studies (Muletz et al., 2012), as probiotic, through bioaugmentation. As Bletz et al (2013) defined it "…bioaugmentation is the augmentation of locally occurring protective bacteria to an individual or the environment with the purpose of altering the hosts' microbial community structure to mitigate disease".

In general, the immunity of organisms is put to the test when they are facing or exposed to xenobiotics since it becomes weakened. From a weakened immune system, infections might occur and with them alterations in the homeostasis. The effects begin at the cellular level but in most cases are detected later when already damage of tissues and organs occurred. Then it becomes more difficult to reverse the situation. One example of an effect is catalytic reactions that can generate overproduction of reactive oxygen species (ROS) (Ochsendorf, 1998). ROS are reactive molecules containing oxygen and they spontaneously react with other molecules. These reduction products of molecular oxygen (O_2) include the superoxide

anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-). On a cellular level and in a controlled way, ROS are produced as the byproducts during the mitochondrial respiratory chain and functions in various physiological processes. Nonetheless, due to exogenous factors such as infections, chemicals, metals or UV radiation their production can increase and lead to protein oxidation, lipid peroxidation or DNA damage (Ochsendorf, 1998; Khansari et al. 2009). The control of ROS is associated with the antioxidant defense mechanisms from enzymatic origin. When an imbalance between antioxidant defenses and the production of free radicals occurs, where there is an ROS excess compare to antioxidant cell response, it is said that the organism is under oxidative stress (Khansari et al. 2008; Ferreira and Abreu, 2007; Apel and Hirt, 2004). To cope with this, living cells have enzymatic antioxidant defenses with enzymes such as glutathione peroxidase (GPX), glutathione reductase (GRED) and glutathione-S- transferase (GST), which functions as a coordinated system in ROS elimination. (Gillardin et al. 2009) and can be used as biomarkers.

Considering that one of the most recent ways to protect amphibians from diseases is through strengthening their immunity with probiotics, and that these should be adapted to each species (Becker et al., 2011), it is essential to search for adequate probiotics and assess their effectiveness. Furthermore, most of the endeavor for finding appropriate probiotics are related with *Bd*, leaving on the side other pathogens, namely bacteria, which may cause also severe health issues on amphibians. Within bacteria, the genus *Aeromonas* is one of the main responsibles for most deaths in amphibians. Taking this into account, the aim of this study was to determine the extent to which bacteria isolated from *P. perezi* with previously tested antimicrobial potential, could protect tadpoles from pathogens such as *A. salmonicida*, making use of antioxidant activity as an indicator of infection.

3.2 Material and Methods

3.2.1. Test organisms

P. perezi eggs were collected in the 8-9th stage of development (Gosner, 1960) (Fig.1 and 2) in a segment of Vouga River located in Almargem Fluvial Beach and previously used as a reference site in other studies (Marques et al., 2013). Afterwards, they were placed in containers with water from the local river and transported to the laboratory. After arrival they were placed into tanks in a climatic chamber until the beginning of the assays. During this time the tadpoles were not fed. When they reached the 20th Gosner stage, and with the help of a pipette, they were removed and placed in glass beakers.

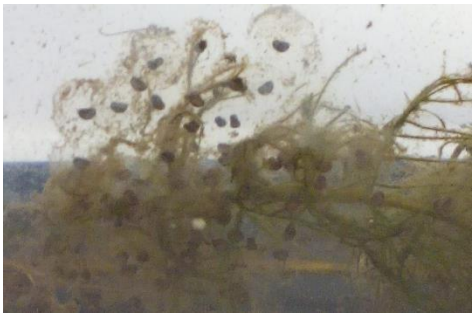


Fig.1 and 2 – *Pelophylax perezi* embryos in FETAX medium, before the beginning of the assay.

3.2.3 Bacterial cell culture

Bacteria used in this study (*P. rhizosphaerae*, *P. fluorescens*, *B. mycoides*, A. *salmonicida* and *E. coli* ATCC 25922) were previously cultured for 18 h in 50 ml Falcon tubes with TSB medium. Then they were centrifuged at 4 000 G and supernatant was removed. Resuspension of the bacteria was made by the addition of 50 ml FETAX (previously sterilized with Whatman 0.2 µm filter) and the centrifugation step at 4 000G was repeated and the supernatant discarded. This step was made to remove any TSB

residues that remained in the pellet. The resuspension of the pellet was made again with FETAX and, to standardize each inoculum, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard, was used. For an absorbance value of 0.008 to 0.1 determined spectrophotometrically at 625 nm the suspension was considered to contain approximately 1 to $2x10^8$ CFU/ml.

3.3 Experimental assay

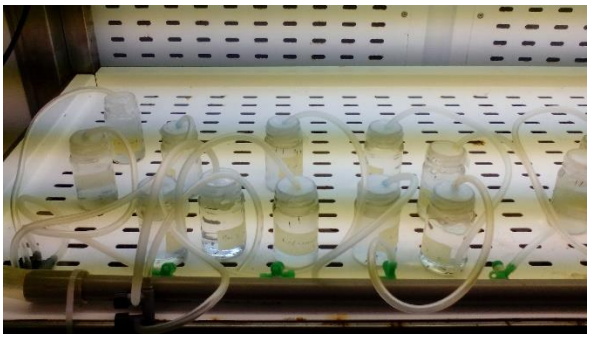
Based on the results obtained in the bacterial growth inhibition assays, described in chapter II, the three bacteria presenting higher antimicrobial activity (*P. rhizosphaerae*, *P. fluorescens*, *B. mycoides*) were selected to test their activity *in vivo* in *P. perezi* tadpoles. Tadpoles were exposed to bacterial solutions containing both bacteria with antimicrobial activity, as well as, the pathogenic bacteria *A. salmonicida* and a standard bacteria (*E. coli*). The assay was carried out at a constant temperature (20 ± 1 °C) and photoperiod (16 hL: 8 hD) conditions. Animal mortality was checked every day and dead animals were removed from the medium. Abiotic parameters, such as pH (WTW330/SET-2 pH meter), dissolved oxygen (WTW315i/SET Oxi meter) and conductivity (LF 330/SET conductivity meter) were recorded at the beginning and at the end of the assay. The experimental assay ended when all tadpoles in the control reached the 25^{th} Gosner stage.

In the table and images below (Tab.1; Fig 3 and 4) is illustrated the experimental design. Ten conditions (A-J) were tested, with three replicas. Each replica consisted of 10 tadpoles placed in a glass vial with 50 ml of the test solution. Condition A is the control where tadpoles were placed into filter sterilized FETAX (Dawson and Bantle, 1987) solution without any bacteria added. Under the conditions G, H, I and J were added equal proportions of volume of bacterial isolates with antimicrobial activity and *A. salmonicida* so the number of bacterial cells was between 1 to 2×10^8 CFU/ml. At the end of the assay the total size of the tadpoles (head to tail) was measured, using an Olympus SZX9 stereoscope. Also abnormalities and deaths were registered. Afterwards, tadpoles were washed in a clean FETAX solution without bacteria and

frozen in liquid nitrogen in Eppendorf tubes and stored at -80 ºC for further biochemical analyses. For the biochemical analyses the tadpoles from each replica were pooled together.

Table 1. Experimental assay

Condition	
A	Without bacteria
B	*Pseudomonas rhizosphaerae*
C	*Pseudomonas fluorescens*
D	*Bacillus mycoides*
E	*E.coli*
F	*A.salmonicida*
G	*A.salmonicida + Pseudomonas rhizosphaerae*
H	*A.salmonicida + Pseudomonas fluorescens*
I	*A.salmonicida + Bacillus mycoides*
J	*A.salmonicida + E.coli*



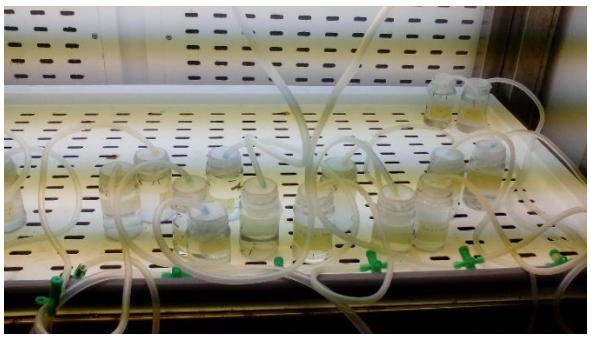


Fig. 3 and 4 – Experimental set during the *in vivo* exposure to bacterial solutions.

3.4 Biomarkers

The activity of enzymes from the antioxidant defense system, namely, total GPX, selenium-dependent GPX, GRED and GST, was used to assess the status of the tadpoles after the assay. Furthermore, lipid peroxidation was also determined through the measurement of the thiobarbituric acid reactive substances. Tadpoles previously preserved in liquid nitrogen were homogenized in ice-cold phosphate buffer (50 mM,

pH = 7.0 with 0.1% Triton X-100). Then were centrifuged at 15 000 g for 10 minutes at 4 ° C. Supernatants were divided into 5 aliquots for protein and each biomarker determination (GRED, GPX, GST, TBARS) and stored at -80 ° C until used.

GRED (EC 1.8.1.7) enzymatic activity was determined according to Carlberg and Mannervik protocol (1985). Briefly, the activity was monitored spectrophotometrically by following the NADPH oxidation at 340 nm (molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$). GST (EC 2.5.1.18) activity was determined according to Habig et al. (1974) following the increment of absorbance at 340 nm, which results from the formation of a thioeter (molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$) due to the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, catalyzed by GST. GPX (EC 1.11.1.9) activity was determined according to Flohé and Günzler (1984) and it was monitored by the oxidation of NADPH at 340 nm (molar extinction coefficient of 6.2 $mM^{-1}cm^{-1}$) where GSSG is reduced back to GSH by the GPX action. This enzyme activity was assessed using H_2O_2 (0.255 mM) and cumene (0.7 mM) as substrates corresponding to selenium-dependent glutathione peroxidase and total glutathione peroxidase respectively. Lipid peroxidation was measured by the quantification of TBARS according to Buege and Aust (1978). This determination is based on the reaction of lipid peroxidation by-products such as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA) measured spectrophotometrically at 535 nm (molar extinction coefficient of 1.56x105 $M^{-1}cm^{-1}$). Protein content was determined spectrophotometric to 595 nm according to the method of Bradford (1976).

3.5 Statistical analysis

To test for significant differences between tadpoles' sizes and between the antioxidant enzymatic activities and TBARS, the data was analyzed through parametric one-way analysis of variance (ANOVA) followed by a Tukey test. The statistical software used for the statistical analysis was Statistica7.0. All data was checked for normality and homogeneity to meet statistical demands (Zar 1996). Whenever the ANOVA assumptions were not verified, a non-parametric Kruskal-Wallis test for

multiple comparisons was performed. In the case of the total GPX and GPX selenium-dependent it was necessary to proceed with data transformation through the function log10 in order to comply with the ANOVA assumptions.

3.6 Results

At the end of the assay mortality was only relevant in condition F (*A. salmonicida*) with 33 % of dead tadpoles. On the other conditions mortality was negligible being lower than 4 %. The development stage also did not present differences between the tested conditions, not even in the tadpoles exposed to the pathogenic agent, reaching all the 25^{th} development stage (Gosner, 1960). Also, no abnormalities were visible in any tadpole. The total body length of the tadpoles (Fig. 5) didn't present significant differences between the tested treatments. Nonetheless the conditions F and G (*A. salmonicida* and A. salmonicida + *P. rhizosphaerae*, respectively) presented slightly lower body lengths.

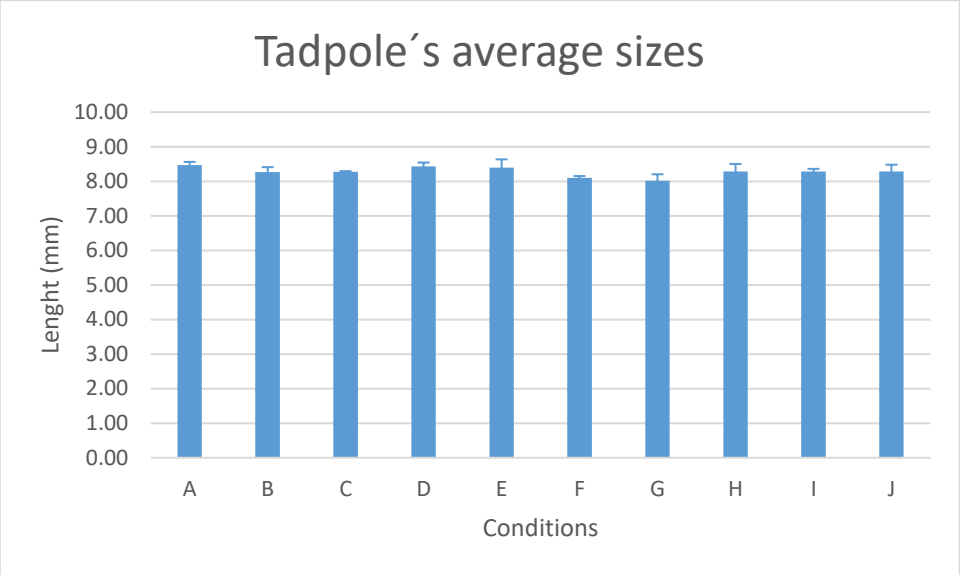


Fig.5 Mean tadpole´s body length after exposure to the respective tested conditions. Error bars represent standard error.

Concerning enzymatic activity despite not having significant differences between treatments, and with few exceptions, there was a tendency in total GPX (Fig. 6) and selenium-dependent GPX (Fig. 7) for lower activities in tadpoles exposed to single bacteria, when comparing to the respective exposure with the pathogen. As for GST (Fig. 8) and GRED (Fig. 9), there was no visible pattern associated with the treatments. Nonetheless, for GST the activity of the tadpoles exposed to *A. salmonicida* (condition F) there was a clear higher activity. Overall, with the exception of selenium-dependent GPX and for some treatments in GRED, the tadpoles exposed solely to *A. salmonicida* (condition F) presented a tendency to have higher enzymatic activity. As for lipid peroxidation, the TBARS values (Fig.10) were globally higher in exposures with single bacteria. Considering only the treatments where tadpoles were exposed to bacteria, the higher values were obtained for the tadpoles exposed to *Pseudomonas* genus and also to the pathogenic agent *A. salmonicida*.

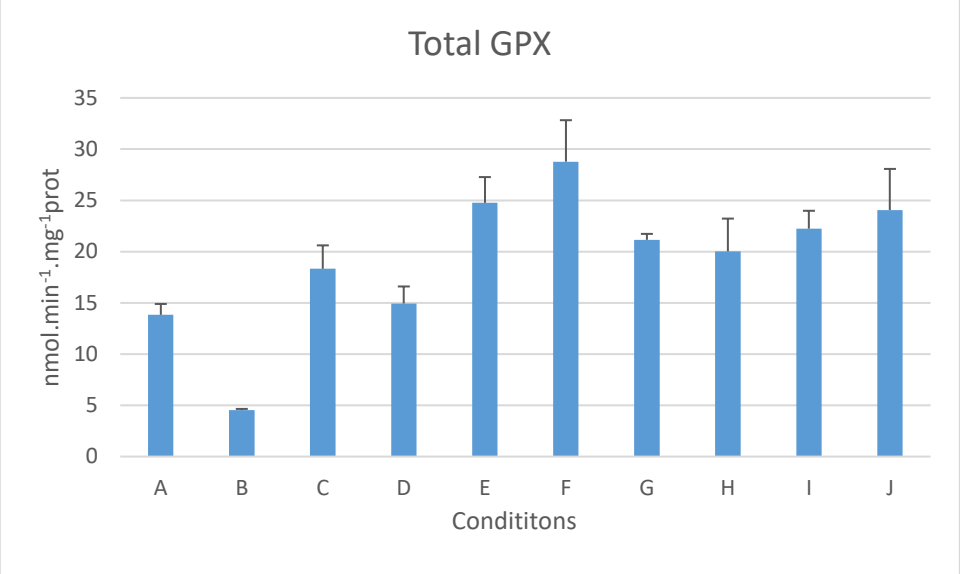


Fig. 6 Mean total glutathione peroxidase (Total GPX) activity (nmol.min^{-1}.mg^{-1}.prot). Error bars represent standard error.

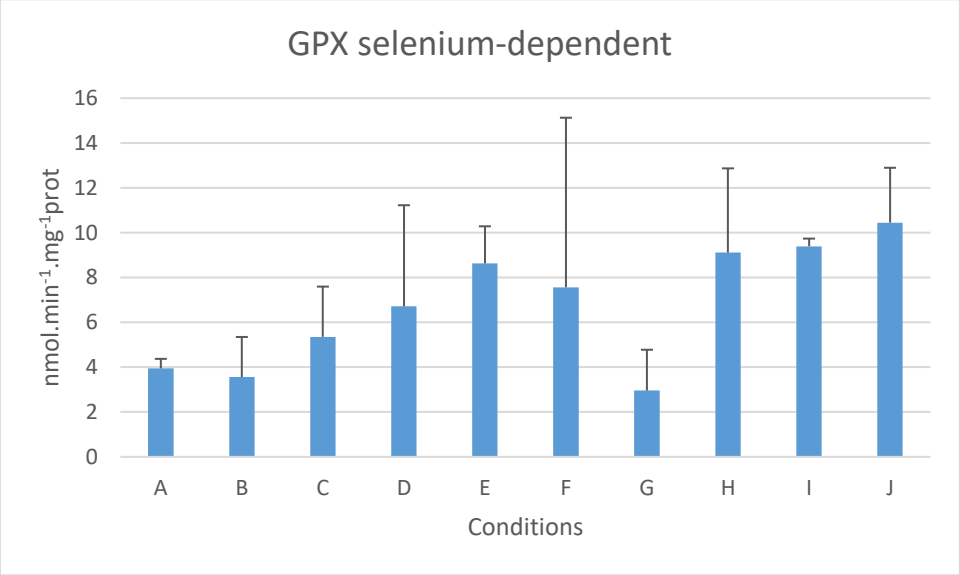


Fig. 7 Mean selenium-dependent glutathione peroxidase (GPX) activity (nmol.min^{-1}.mg^{-1}.prot). Error bars represent standard error.

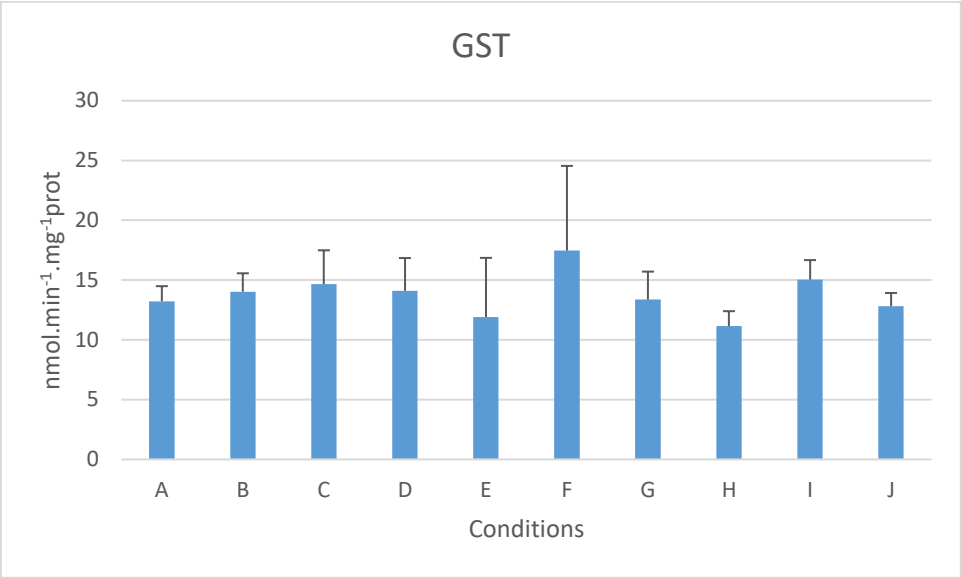


Fig. 8 Mean glutathione-S-transferases (GST) activity (nmol.min^{-1}.mg^{-1}.prot). Error bars represent standard error.

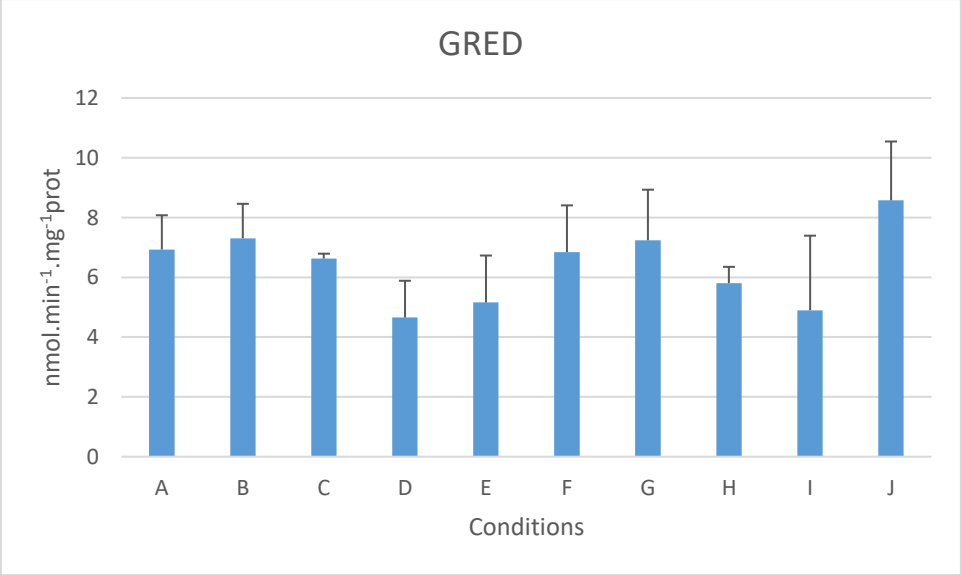


Fig. 9 Mean glutathione reductase (GRED) activity (nmol.min^{-1}.mg^{-1}.prot). Error bars represent standard error.

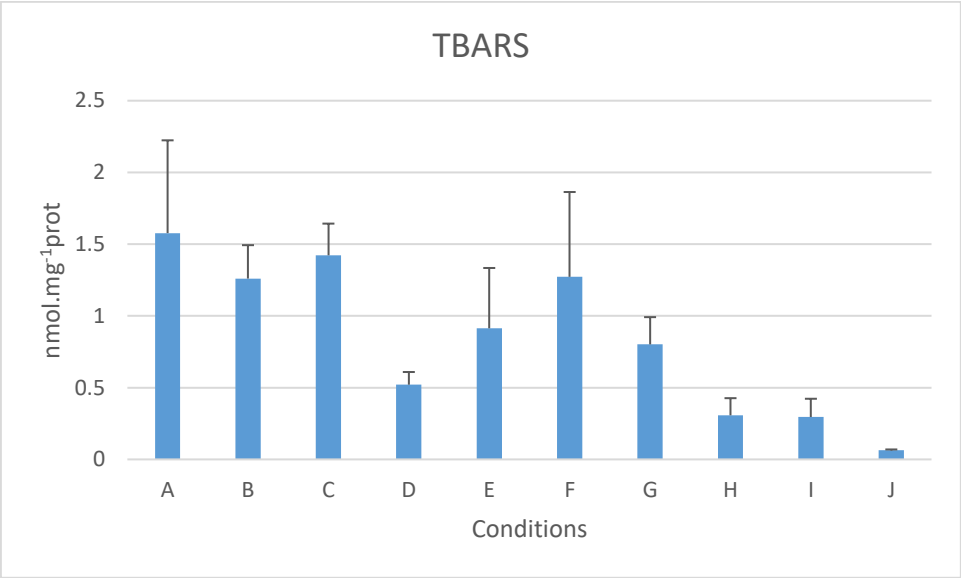


Fig.10 Mean content of thiobarbituric acid reactive substances (TBARS) (nmol.mg^{-1}.prot). Error bars represent standard error.

3.7 Discussion

The decrease in amphibian populations due to pathogenic agents is one of the main present concerns and many have been the studies addressing this subject (Becker et al., 2009, 2011; Bletz et al., 2013; Lauer et al., 2007). The great majority of them are righteously focused on *Bd* since it is widely spread and lethal. Nonetheless other pathogens, namely bacteria from the genus *Aeromonas*, despite their known negative effect on frogs (e.g. Hill et al., 2010), are still far from being entirely studied, namely concerning interactions with other bacteria, including with antimicrobial potential. Our studied is, to our knowledge, the first to test, in amphibians, the potential probiotic action against bacteria from the *Aeromonas* genus. Considering the parameters assessed, mortality was one of those which presented bigger differences. From the tested conditions mortality reached 33% in the tadpoles exposed to *A. salmonicida*. Such results indicate a potential for this bacteria to be lethal to this

amphibian species. Nonetheless for other parameters such as growth, there was no clear difference between conditions tested. Despite having a slightly lower body size in tadpoles exposed to the pathogen, the differences where far less pronounced than one could eventually expect. In animals with no chance of acquiring energy from sources other than their yolk, it could be expected for them to have interference in the allocation of their energy, impairing thus their growth or even development. Nonetheless, the information available to compare our results is scarce and one of the few studies focusing on tadpoles and the effect of a pathogen was made with the aim of accessing their foraging ability (Venesky et al., 2009). However, in *P. perezi* tadpoles there is a study that shows that, in the same larval stages as the ones used in our study, contaminated effluents affect growth, suggesting metabolic costs as one reason for such results (Marques et al., 2008). Regarding the values of the lipid peroxidation damage in the graphic of TBARS, it was observed that in the control (A), where the tadpoles were exposed only to FETAX, the values were among the highest between the remaining conditions. Nonetheless, when comparing the TBARS control results obtained by Marques et al. (2013) for in situ assay with our own, there are no remarkable differences between them. This indicates that apparently the controls where within normal range of lipid peroxidation levels, and that in fact the exposure to bacteria led to lower peroxidative values. It is important to note that in general, the values of the enzymatic activities were high for condition F (tadpoles exposed to the pathogenic bacterium *A.salmonicida*). These results when comparing with similarly high enzymatic values (e.g. condition I and J) would lead us to expect similar results also in the lipid peroxidation damage, which in fact does not occur. Indeed the tadpoles exposed to condition F present higher damage than the ones from condition I or J. This may indicate that even though there is a higher activity of enzymes such as Total GPX or GST, the antioxidant defense system isn't able to lower lipid damage as it is observed in conditions I and J. In addition, the TBARS values of the probiotic bacteria did not register values below those of the control being, with exception for the condition D (tadpoles exposed to *Bacillus mycoides*). The results obtained in this study seem to indicate that when the probiotic bacteria are together with the pathogenic bacterium

A.salmonicida, there is an activation of its antioxidant enzymes. This may be related to the "oxidative burst" observed when there are infections or contact with contaminants (Albert et al. 2007; Froese et al. 2005). This may lead to an increase in the activity of antioxidant enzymes and if this antioxidant activity is efficient a decrease in ROS levels and a consequent reduction of oxidative damages may occur. In summary, the exposure of bacteria with antimicrobial potential with pathogenic bacteria, as in this case *A.salmonicida*, with similar levels of cell density in all treatments, tends to cause a tendency of increase of the antioxidant activity resulting in a decrease of the peroxidative damages. Overall the three tested probiotic agents presented good results in terms of reduction of peroxidative damage and of avoiding mortality. Within these agents *B. mycoides* seems to be the one with the higher ability of stimulating the antioxidant enzymes, which in turn results in lower peroxidative damage on both single exposure and simultaneous exposure with *A. salmonicida*.

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Chapter IV
Final Remarks

4. Final Remarks

This last chapter aims to provide a general perspective of the two previous chapters (II-III) integrating the results according to the main objectives of this thesis. Considering that amphibians have been declining in the last few decades, with diseases playing a major role in this factor (Stuart et al., 2004), and that, as reviewed by Allentoft and O'Brien (2010), genetic diversity is essential to maintain the levels of fitness in amphibian populations, this work focused on studying cultivable bacteria from the skin of *P. perezi* from two urban zones and their potential to protect the animals against pathogens. Usually animals from contaminated and urban areas are disregarded as animals with potential to contribute for the genetic pool of a species, nonetheless these areas can sometimes be the bridge connecting populations from different uncontaminated or rural areas, allowing gene flow and avoiding the complete isolation. Thus, and specifically for amphibians it is also essential to assure the viability of these populations many times weakened by contamination or stress (Schadich et al., 2009). Bearing this in mind this work has focused primarily on the study of cultivable skin microbiota from *P. perezi* from two urban sites in different seasons, autumn and spring, and verify if some of the bacterial isolates could show antimicrobial activity. The next step was, from the isolates that demonstrated the highest antimicrobial activity, see if they could protect tadpoles from pathogens such as *A. salmonicida* using the enzymatic activity as an infection indicator. For the first objective, the chosen sites were river beach Olhos de Fervença in the city of Cantanhede and the St. António Park located in the city of Aveiro. Bacteria were isolated from both females and males. It was found that there were differences in cultivable microbiota depending on the location, sex and season. Most of the bacterial diversity was found in St. António Park. There were also isolates that were different between seasons. Regarding the antimicrobial activity, was observed that from a total of 120 bacterial isolates, 19 showed to have antimicrobial activity. This was predominantly observed in river beach Olhos de

Fervença, in the autumn and especially in females. These results indicate that there is a great site and season influence in terms of cultivable microbiota and antimicrobial activity, which reinforces the previously pointed by Flechas et al. (2012). From the 19 bacterial isolates, 3 showed higher antimicrobial activity. These three were used in the experimental assay on the Chapter III to test if they were able to protect tadpoles from pathogens such as *A.salmonicida* reducing the oxidative damage. The experimental assay was based on the exposure of tadpoles to different solutions containing the bacteria with antimicrobial activity and *A. salmonicida* or the two together. The results showed that in general the exposure to *A.salmonicida* showed higher values of enzymatic activity. In addition, the TBARS values for the probiotic bacteria were not below those of the control, being the exception tadpoles exposed to *Bacillus mycoides*. The results obtained in this study seem to indicate that when the probiotic bacteria were together with *A.salmonicida*, there is an activation of its antioxidant enzymes. This could result in a reduction of oxidative damage. This might show that the probiotic bacteria previously isolated from *P.perezi* were effective in reducing the peroxidative damage in tadpoles and could be used as probiotics and in bioaugmentation techniques protecting the amphibians from pathogens and leading to the preservation of some species. Future work needs to be done, for example, testing these probiotic bacteria in other pathogens like *Bd* fungus or in against other pathogenic bacteria. It's important to verify if the bacteria from frog´s skin can or cannot protect effectively these animals taking into account that they are from areas with anthropogenic influence. Also in future, these bacteria could be tested in adult individuals from different species. Another future study would be extending the time of the experimental assay by leading the development of tadpoles reach higher stages comparatively to the Gosner stage $25^{th,}$ as this could influence the results at the level of the antioxidant enzymatic activities.

In conclusion, there are bacteria from the amphibians with antimicrobial activity against pathogenic bacteria, being however site specific and presenting seasonal variations. These bacteria can be used in future studies as probiotic agents and may contribute to the preservation of species.

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